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14. ABSTRACT
Using an animal model of prostate tumor progression in the bone we have previously shown that MMPs, namely MMP-2,-3,-9 and -13, are overexpressed at the tumor bone interface and these MMPs are for the most part expressed by the host cells of the bone. To test the contribution of MMPs in prostate tumor progression in the bone, we have generated mice that are immunocompromized and deficient for MMP-2,-3 and -9 during the current period. We have found that MMP-9 does not contribute to prostate tumor progression in the bone since no difference in osteolytic or osteoblastic responses between wild type and MMP-9 deficient animals were detected by Faxitron, CT, SPECT and histomorphometry. These results, while negative, are important for the generation of selective MMP inhibitors that lack the deleterious side effects associated with broad spectrum inhibitors. In addition, we have also identified PTHrP as an MMP substrate and postulate that MMP processing of PTHrP may be a mechanism through which MMPs can contribute to tumor induced osteolysis.

15. SUBJECT TERMS
Osteolysis, osteoblastic changes, prostate progression in bone, matrix metalloproteinases, MMPs, receptor activator of nuclear kappa B ligand, RANKL, bone metastasis

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Introduction

This year, in the United States alone, of the 27,350 men who die from prostate cancer, 80% will have evidence of bone metastasis (1, 2). Prostate bone metastases cause several complications for patients such as hypercalcemia, spontaneous bone fracture and debilitating pain that dramatically affects their quality of life. To progress in the bone, the invading prostate tumor cells induce radical changes in bone matrix homeostasis by stimulating osteoblastic and osteolytic changes (3). These changes result in an actively remodeling bone tumor microenvironment, rich in mitogenic signals that promote tumor growth. In turn, the growth of the tumor exacerbates the osteoblastic and osteolytic changes in a manner that has been well described as the 'vicious cycle' (4). Using an animal model of

tumor progression in the bone, we have previously identified a group of enzymes known as matrix metalloproteinases (MMPs) as being highly overexpressed at the tumor bone interface in comparison to the tumor area alone. In a bid to understand the importance of these MMPs, namely MMP-2, -3, -9 and -13, in prostate tumor progression in the bone, we aim to generate MMP null animals and compare those animals to their wild type counterparts. While the MMPs are important in the turnover of the extracellular matrix, it has become apparent that the MMPs are also capable of regulating cell:cell communication by processing various cytokines and growth factors to active soluble forms (5). These soluble factors often influence biological processes including survival, proliferation, angiogenesis and osteoclast activation. Therefore, understanding which MMPs are important in contributing to prostate tumor progression in the bone and identifying the mechanisms that govern the vicious cycle can provide valuable targets for therapeutic development.

Body……

Accomplishments

Aim 1. *Determine the stromal contribution of MMPs that are markedly overexpressed at the tumor:bone interface namely, MMP-2,-3,-9 and -13 to prostate cancer induced osteoblastic and osteolytic changes in the bone*.

a) Generate immunocompromised RAG-2$^{-/-}$ mice that are deficient in MMP-2, MMP-3 and MMP-13 by crossing RAG-2$^{-/-}$ mice with MMP$^{-/-}$ mice that are both available on the C57Bl/6 background (Months 1-12).
b) Using our pre-clinical animal models, we will test the contribution of stromal MMP-9 to tumor induced osteoblastic and osteolytic change in readily available immunocompromised RAG-2$^{-/-}$ MMP-9 deficient mice (Months 1-12).
c) Test the contribution of stromal MMP-2, MMP-3 and MMP-13 to tumor induced osteoblastic and osteolytic change using our pre-clinical model (Months 11-30).

d) Identify the expression of stromal MMPs in human clinical samples of prostate bone metastases (Months 20-36).

The proposed animal model in the current program involves the transplantation of moderately differentiated rat prostate adenocarcinoma to the calvaria of immunocompromised wild type and MMP deficient mice. To achieve this, we proposed to cross C57Bl/6 RAG-2 (recombinase activating gene-2) deficient mice with either C57Bl/6 MMP-2, -3 or -13 deficient animals in order to generate F2/F3 animals that are immunocompromised and deficient for the desired MMP. As of January 2009, we have generated RAG-2$^{-/-}$;MMP-2$^{-/-}$, RAG-2$^{-/-}$;MMP-3$^{-/-}$ and RAG-2$^{-/-}$; MMP-13$^{-/-}$ animals and have made preliminary observations with these animals in terms of prostate tumor induced osteolytic and osteoblastic changes.

Host MMP-9 impacts angiogenesis in the prostate tumor-bone microenvironment but does not impact tumor induced osteolytic and/or osteoblastic changes.

In the initial 12 months of the project, the PI focused on assessing the impact of host derived MMP-9 in tumor induced osteolytic and osteoblastic changes. In repeated studies, with at least 10 animals per group, we determined that MMP-9 does not have any effect on tumor progression in the bone using both whole animal imaging modalities such as microCT and microSPECT and traditional histomorphometry approaches. While there is a trend

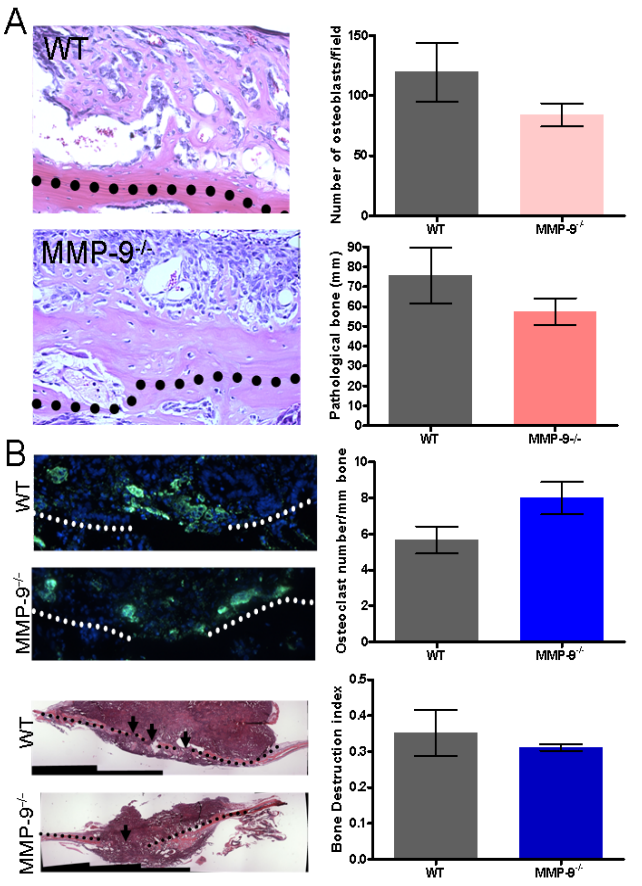

Fig. 1. Host MMP-9 does not contribute to prostate tumor induced osteolytic or osteoblastic changes. **A:** The amount of pathological bone (dashed line) was assessed in sections from WT (n=24) and MMP-9 deficient (n=37) animals at week 3. The number of osteoblasts generating pathological bone was also measured. **B:** The numbers of TRAP positive multinucleated osteoclasts (green) at the tumor bone interface (dashed line) in multiple sections revealed no difference in numbers between the WT and MMP-9 deficient groups. DAPI (blue) was used as a nuclear stain. No differences in the bone destruction indices were observed. Arrows indicate areas of osteolysis.

towards a decrease in osteolysis in the MMP-9 deficient animals, this decrease has not proven to be statistically significant (Figure 1). While these data suggest that MMP-9 does not contribute to tumor progression in the bone, it should be stated that the role of MMP-9 in 1) the metastasis of prostate cancer to the bone or 2) in the initial survival/establishment of the prostate tumor cells in the bone microenvironment can not be ruled out since these steps are not recapitulated in our animal model. Previous studies have shown that MMP-9 is important in mediating angiogenesis in the tumor microenvironment (6) and using the endothelial antigen CD-31 as a marker for angiogenesis, we have found decreased angiogenesis in the MMP-9 null group (Figure 1). We are currently preparing a manuscript documenting our findings.

Host MMP-13 has a protective role in the prostate tumor-bone microenvironment

Murine MMP-13 is considered to be the ortholog of human MMP-1, and MMP-13 deficient animals have been reported as having a delay in endochondral ossification during skeletal development with thickened trabecular bone persisting in the animals (7, 8). In collaboration with Dr. Stephen Krane, the PI has generated immunocompromised MMP-13 deficient mice. At this juncture, histological analysis of the calvaria from these animals at 6 weeks of age and their age matched wild type counterparts appear to be similar with respect to the amount of bone and number of osteoclasts (data not shown). Surprisingly, preliminary studies using our

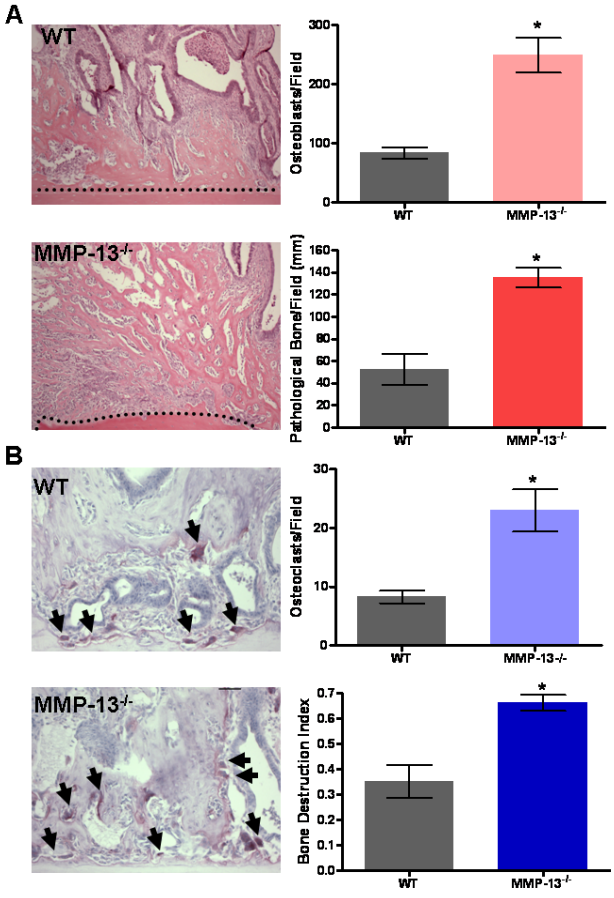

Fig. 2. MMP-13 has a protective role in prostate tumor induced osteoblastic and osteolytic changes. **A:** The number of osteoblasts and the amount of pathological bone in multiple sections (10x fields) were calculated in MMP-13 (n=7) and wild type mice (n=5). **B:** The number of TRAP stained (red) multinucleated osteoclasts (arrows) per field in addition to the to the bone destruction index in multiple sections were also calculated. Asterisk denotes statistical significance with $p<0.05$.

animal model suggest that host MMP-13 plays a protective role in preventing tumor induced osteolytic and osteoblastic changes (Figure 2). At three weeks post implantation, immunocompromised wild type and MMP-13 deficient animals were sacrificed. Histological analysis revealed that in comparison to wild type controls, MMP-13 deficient sections had higher numbers of osteoblasts and osteoclasts which was consistent with increase bone formation and destruction respectively. These data are unexpected since MMP-13 has been described as the rate limiting collagenase for murine skeletal development. Our data, while very preliminary, suggests that in the pathological context of the tumor bone microenvironment, *host MMP-13 inhibits the vicious cycle and plays a protective role in the tumor-bone microenvironment*. The concept of MMPs as playing a protective role during tumor progression has been reported for MMP-3 and MMP-8 during skin carcinogenesis and progression in mice but to our knowledge MMP-13 has not been described as being protective in a pathological context (9, 10). These studies will be repeated again and the molecular mechanisms through which MMP-13 protects against prostate tumor progression in the bone will be a primary focus over the final year of the proposal

Thus far, our data illustrate that MMP-7, -9 and -13 are highly expressed in the prostate tumor-bone microenvironment but on an individual basis, they clearly play very different roles with respect to tumor induced osteoblastic and osteolytic changes. We are currently evaluating the contribution of host MMP-2 and MMP-3 in our model. These preliminary findings support the rationale for delineating the individual roles of MMPs in the pathological context of the prostate tumor-bone microenvironment so that selective inhibitors can be generated that lack the deleterious side effects of broad spectrum inhibitors. Further, identification of the mechanisms through which MMPs act to contribute to or protect against tumor induced osteolytic and osteoblastic changes may also reveal new therapeutic strategies for the treatment of prostate to bone metastases. Therefore, Specific Aim 1 will determine the contribution of MMP-2 and MMP-3 to prostate tumor induced osteolytic and osteoblastic changes while fully exploring the potential protective effect of host MMP-13.

To determine the clinical relevance of host MMPs in tumor progression in the bone, we have been collecting non-identified samples of human prostate to bone metastasis. Through the Department of Orthopaedics and Rehabilitation here at Vanderbilt, we have collected a limited number of samples that will not give us the statistical power necessary to determine the prevalence of MMP expression in human prostate to bone metastases. Therefore, the PI has begun a collaboration with Dr. Bob Vessella, Dept. of Urology, University of Washington. Dr. Vessella has agreed to provide human samples of prostate to bone metastasis (up to 800 if necessary) collected through his rapid warm body autopsy program at the University of Washington (11, 12). The localization of MMPs in these samples will be performed over the final 12 months of the project.

Aim 2. *Identify and test MMP processed substrates that mediate prostate tumor induced osteolytic and osteoblastic change.*

a) Identify and test candidate MMP substrates that mediate prostate tumor induced osteolytic and osteoblastic change.
b) Determine the contribution of MMP solubilized RANKL vs. MMP resistant RANKL in mediating osteoclastogenesis

In aim 2, we have taken a candidate approach in a bid to identify the potential factors that MMPs process in order to mediate tumor induced osteolysis. Bone is a rich reservoir of growth factors such as transforming growth factorβ (TGFβ) and insulin like growth factor-1 (IGF-1) and both of these factors have been implicated in driving the 'vicious cycle' (4). Given that various MMPs have been reported as processing the molecules that keep these growth factors in a latent state such as latency TGFβ binding proteins (LTBPs) and IGF binding proteins (IGF-BPs) we envisage that these would be excellent candidate molecules through which MMPs could contribute to osteolytic and osteoblastic effects. Given the labile nature of TGFβ we have taken multiple approaches into identifying the activity status of the cytokine in wild type and MMP null animals. Using procedures described by

Barcellos-Hoff et al.(13), we have been able to generate frozen sections of non-decalcified bone using the Cryo-Jane Tape transfer system. This approach allows us to visualize latent and active TGFβ in the tumor-bone microenvironment using microscopy (Figure 3). We have also examined the presence of active TGFβ using ELISA. We have observed that there is more active TGFβ at the tumor bone interface in comparison to the tumor area alone in wild type animals but thus far have found no difference in the levels of active TGFβ between wild type, MMP-7 and MMP-9 null animals (Figure 3).

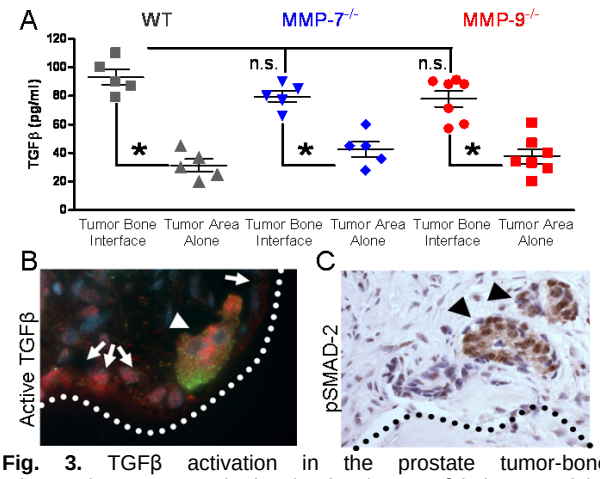

Fig. 3. TGFβ activation in the prostate tumor-bone microenvironment. **A.** The levels of active TGFβ in lysates of the tumor-bone interface and tumor area alone derived from wild type (WT), MMP-7 and MMP-9 deficient animals were measured by ELISA. Asterisk denotes statistical significance ($p<0.05$) while n.s. denotes statistical non-significance. **B.** Immunofluorescent detection of active TGFβ at the tumor bone interface (red; arrows) in frozen sections. Dashed line indicates tumor bone interface while arrow head indicates TRAP positive (green) multinucleated (DAPI;blue) mature osteoclasts. **C.** pSMAD-2 (arrow head; brown), a down stream effector of TGFβ signaling, was detected in cancer cells proximal to the tumor-bone interface.

Using these approaches and following up with western blot and immunolocalization studies, we have the ability to rapidly assess if host derived MMPs, such as MMP-2, -3 and -13 are playing a role in the release of active TGFβ from the bone.

In the metastatic bone:tumor microenvironment, parathyroid related hormone (PTHrP) has been identified as a powerful mediator of osteolysis (14). Pro-PTHrP has three isoforms that are 139, 141 or 173 amino acids in length. These isoforms are subsequently enzymatically processed to yield the mature form of $PTHrP_{1\text{-}36}$ (amino acids 1-36). Thus far the enzymes implicated in generating mature PTHrP have been; endothelin converting enzyme-1 (ECE-1); ECE-2 and neprilysin which are not MMPs but are members of the metazincin family of proteinases. Interestingly, prostate

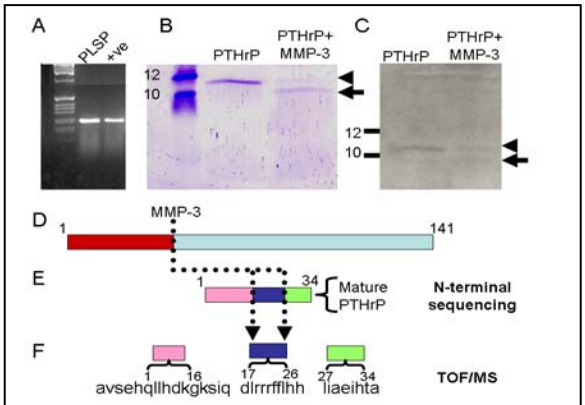

Fig. 4. PTHrP is an MMP substrate. **A:** RT-PCR analysis of PTHrP expression by the prostate adenocarcinoma tissue (PLSP). +ve refers to a positive control. **B:** Mature PTHrP (1µg; arrow head) was incubated for 1 hour at 37°C either alone or with 100ng of active MMP-3 (50mM Tris, 0.1M NaCl, 5mm $CaCl_2$). Arrow indicates MMP-3 cleavage product. Markers are in kDa. **C:** Immunoblot analysis also demonstrates PTHrP processing using an antibody directed to the C-terminus (Santa Cruz; sc-20728). **D-E:** N-terminal amino acid sequencing revealed that MMP-3 processing occurred between amino acid 34 and 35 to yield $PTHrP_{1\text{-}34}$. **F:** MALDI-TOF MS showed that MMP-3 was capable of further processing of $PTHrP_{1\text{-}34}$. The amino acid sequence of the fragments is also illustrated. Similar analyses revealed no degradation of full length PTHrP in the absence of MMP-3.

specific antigen (PSA) which is a serine protease has also been shown to process PTHrP but in a different region that generates a 23 amino acid form of $PTHrP_{1-23}$ (Cramer et al., 1996). This is thought to abolish the activity of the hormone but some studies suggest that smaller molecular weight versions of PTHrP can have differential effects compared to $PTHrP_{1-36}$ (15).

Since PTHrP can be processed by members of the metazincin family and given the presence of MMPs in the tumor bone microenvironment, we asked whether MMPs could process PTHrP. Using recombinant $PTHrP_{1-141}$, we observed that MMP-3 and MMP-7 generate mature $PTHrP_{1-36}$ (Figure 4). Further examination by mass spectroscopy revealed that MMP-3 and MMP-7 further processed $PTHrP_{1-36}$ into smaller fragments, namely $PTHrP_{1-16}$, $PTHrP_{17-26}$ and $PTHrP_{27-34}$. A number of the MMP generated PTHrP products have reported cellular functions. For example, $PTHrP_{1-16}$ has sequence similarities to endothelin-1 (ET-1). ET-1 has been identified as a major factor involved in promoting osteoblastic responses via the ET_A receptor (16). $PTHrP_{1-16}$ has been shown to bind to ET_A in cardiomyocytes but apparently has no impact on ET_A signaling when overexpressed in CHO cells (17, 18). However, the precise role of $PTHrP_{1-16}$ in osteoblast function is unclear. Other MMP-3 generated fragments such as $PTHrP_{27-36}$ can mediate protein kinase C signaling via the PTHR-1 receptor (19) but again, the precise role of this fragment in addition to $PTHrP_{17-26}$ in osteoblast function remain unclear. *Therefore, delineating the role of these MMP generated PTHrP products on osteoblast behavior will be a major goal over the course of the remainder of the project.*

We have previously demonstrated that membrane bound receptor activator of nuclear κB ligand (RANKL) which is essential for osteoclast maturation and activation is sensitive to shedding from the cell surface by MMP-3 and MMP-7 (20).

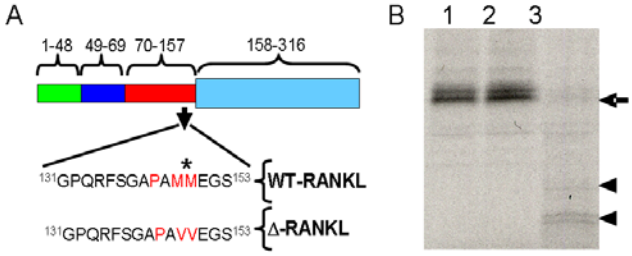

Fig. 5. A: MMP-7 processes RANKL in the juxtamembrane region at amino acid position 142 (see figure 10 for additional information). To generate a non-cleavable mutant RANKL, the methionines at positions 142 and 143 in addition to the proline at position 140 were mutated by single nucleotide substitution using a site directed mutagenesis kit (Stratagene). Methionine to Valine (ATG to GTG) and Proline to Leucine (CCA to CTA) **B:** Wild type and Δ-RANKL full length cDNAs were in vitro translated using the Promega TnT *in vitro* translation system with S^{35} labeled methionine. **1:** Δ-RANKL alone. **2-3:** Δ-RANKL(2) or wild type RANKL(3) incubated with 100ng of MMP-7 overnight at 37°C. Arrow indicates full length unglycosylated Δ-RANKL at approx 40kDa. Arrow heads denote cleavage products.

We have generated a non-cleavable version of RANKL and are currently testing the ability of the non-cleaved RANKL to stimulate osteoclast activation via direct cell:cell contact (Figure 5).

Key Research Accomplishments……..

- Generated RAG-2;MMP-2, RAG-2;MMP3, RAG-2;MMP-13 null animals
- Observed that host derived MMP-9 does not contribute to tumor progression in the bone but does impact angiogenesis
- Have observed that host MMP-13 plays a protective role in preventing tumor induced osteolytic and osteoblastic changes.
- Have begun the examination human samples of prostate to bone metastasis in collaboration with Dr. Vessella.
- Identified higher levels of TGFβ at the tumor bone interface in comparison to the tumor area alone in wild type animals
- Identified that MMP-3 and MMP-7 are capable of generating mature PTHrP
- Have generated a non-cleavable version of RANKL

Reportable Outcomes…….

Manuscripts

Halpern, JL., Bruni-Cardoso, A., Craig, L, Peterson TE., and Lynch, CC. Host matrix metalloproteinase-9 does not impact tumor induced osteolytic/osteoblastic changes in a model of prostate cancer bone metastasis. Manuscript in preparation.

Presentations

Growth Factor and Signaling Symposium, Ames, Iowa, September, 2008

Joint AACR and Metastasis Research Society Meeting, Vancouver, BC, Canada, August, 2008

Tumor host interaction and angiogenesis meeting, Monte Verita, Ascona, Switzerland, October 2007.

Tumor microenvironment (TMEN) meeting, Vanderbilt University, Nashville, TN, September, 2007.

Conclusion……

In previous studies, we have identified that MMPs, namely MMP-2, -3, -9 and -13 are overexpressed at the tumor: bone interface. In the past, human clinical trials involving broad spectrum MMP inhibitors failed due to dose limiting side effects. The principle reason for the failure was due to a lack of understanding as to how MMPs contribute to tumor progression. In animals models of osteolysis, broad spectrum MMP inhibitors have been successful in preventing tumor induced osteolysis and growth (21-23). Therefore, in order to apply MMP inhibitors in the clinical setting, we must identify the individual MMPs that impact tumor progression in the bone. Our initial studies show that host MMP-9 does not contribute to tumor progression in the bone while MMP-13 appears to have a protective effect. These data therefore support the rationale for identifying the contribution of individual MMPs in the prostate-tumor bone microenvironment so that selective MMP inhibitors can be developed that lack the deleterious affects of broad spectrum MMP inhibitors can be developed. To this end, the PI has begun a collaboration with Prof. Qing-Xiang Amy Sang to test the efficacy of selective MMPIs in the In year 2, we will continue testing the contribution of MMP-2, MMP-3 and MMP-13 to prostate tumor progression and examining whether MMP processing of PTHrP, RANKL or TGFβ are responsible for observed phenotypes in the MMP null tumor-bone microenvironments.

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